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Distribution of Oral *Haemophilus* Species in Dental Plaque from a Large Adult Population

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The periodontal status of maxillary first molars in 284 young adults demonstrating near-health to early disease was evaluated, and supragingival and subgingival plaque samples were collected. Plaque samples were processed anaerobically, enumerated microscopically for bacterial morphotypes, and cultivated on various media to enumerate the microflora. Although haemophili were ubiquitous (recovered in 98.5 and 96.2% of the supragingival and subgingival plaque samples, respectively), 50% of the respective samples had proportions of ≤1.5% and ≤0.33% total *Haemophilus* spp. based on total cultivable microflora. To study the distribution of *Haemophilus* spp., 377 colonies were identified from modified chocolate agar (selective for oral haemophili) from 14 supragingival and corresponding subgingival samples from 14 subjects. The most prevalent species, *Haemophilus parainfluenzae*, was found in significantly higher proportions, based on total haemophili on modified chocolate agar, in supragingival and subgingival samples from teeth with shallower probing depths (≤3.0 mm) versus deeper probing depths (≥3.0 mm). Additional statistically significant findings included *Haemophilus segnis* in higher proportions in supragingival samples from deeper sites, *Haemophilus aphrophilus* in higher proportions in subgingival samples from deeper sites, and *Haemophilus paraphrophilus* in higher proportions in subgingival samples from shallower sites. Scatter diagrams illustrating the bivariate distributions of proportions of haemophili with proportions of dark-pigmented *Bacteroides* spp., spirochetes, and streptococci demonstrated that high proportions of haemophili were never recovered from sites with high proportions of *Bacteroides* spp. or spirochetes. All levels of haemophili, however, were recovered from sites with all levels of streptococci. Two potential systems for interpreting haemophili data were hypothesized for predicting periodontal probing depths. There was highly significant agreement between the two systems. Small but statistically significant correlations were found between the gingival index, probing depth, and attachment level, and proportions of total *Haemophilus* species in the respective samples.

It has been suggested that gram negative microorganisms play a central role in the etiology and pathogenesis of periodontal disease (22, 25, 26). Slots, for example, showed that gram-negative bacteria are present only in low numbers in healthy gingival sites, but that they constitute more than 75% of the cultivable microflora in advanced periodontal lesions, with *Fusobacterium nucleatum* and *Bacteroides gingivalis* being the most prominent (22). Few studies, however, have examined the microbial composition of plaque in subjects in relationship to the periodontal status of a subject, utilizing media that could support the growth of the gram-negative oral *Haemophilus* species. The microhabitat of the genus *Haemophilus* in the human oral cavity has not been studied in a large population, and little is known about the proportions and locations of these bacteria in dental plaques.

Kilian and Schiott studied *Haemophilus* spp. present in 15 presumably supragingival plaque samples (9). However, no description of the method of plaque sampling or the relative periodontal status of the subjects was given. Sims has suggested a lack of association of haemophili reported with periodontal disease based on his observation that there were lower numbers of haemophili in crevicular fluid than in saliva (21). No plaque samples, however, were studied. Kilian et al. have shown that haemophili are early colonizers, along with the oral streptococci, of clean tooth surfaces (8).

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In the present study proportions of oral *Haemophilus* spp. were correlated with a number of clinical measurements. Bivariate distributions of the proportions of *Haemophilus* spp. with the proportions of dark-pigmented *Bacteroides* spp., the proportions of streptococci, and the proportions of spirochetes were also examined. In addition, the haemophili from supragingival and the corresponding subgingival plaques from 14 maxillary first molars were speciated to determine the distribution of the various *Haemophilus* spp. in plaque. The principal objectives of this study were to examine the genus and species of *Haemophilus* within the dental plaques of healthy and near-healthy subjects to: (i) better understand the microhabitat(s) of this indigenous gram-negative organism, (ii) determine whether there is a relationship between those microhabitats, the distribution of oral *Haemophilus* spp., and the periodontal status of sampling sites, and (iii) determine whether oral *Haemophilus* spp. may play a role in the microbial interpretation of the status of the periodontium or the risk of periodontal disease.

MATERIALS AND METHODS

Subjects. A total of 284 males and females between the ages of 25 and 40 were selected from patients presenting themselves at the University of Minnesota School of Dentistry for dental care. Patients were excluded from the study if their teeth had been cleaned within 4 months of the initial visit, had taken antibiotics within the previous 6 months, or were missing either or both maxillary first molars. It is

known that only 1 patient of the 284 had received periodontal treatment.

Plaque sampling and clinical measurements. Individual supragingival plaque samples were obtained from the mesio-buccal surfaces of each maxillary first molar with sterile, nickel-plated, Gracey 11/12 curette tips held in a hemostat. The curette tips with the plaque samples were immediately placed in 4-ml conical vials (American Hospital Supplies, McGraw Park, Ill.) containing PRAS (prereduced anaerobically sterilized) diluent (6) and immediately were transferred to an anaerobic chamber with an atmosphere of 10% H_2–10% CO_2–80% N_2 (Coy Manufacturing Co., Ann Arbor, Mich.) These teeth were then isolated by using cotton rolls and gently dried. Subgingival plaque samples were collected at the mesio-buccal surfaces of the maxillary first molars. Curette tips were inserted as far apically as possible to the base of the crevice. Slight pressure was then applied against the tooth as the instrument was drawn coronally. The curette tips with the plaque samples were immediately placed in vials and processed the same as the supragingival samples.

Clinical measurements were obtained concurrent with the plaque samples. The plaque index (PI) was recorded when the supragingival plaque samples were obtained (12). The PI is a standard index for classifying supragingival plaque on a scale of 0 to 3 with integers which are assigned on the basis of specific clinical criteria. Clinical measures of gingival inflammation, probing depth (PD), and attachment level (AL) were made after collection of the plaque samples. Gingival inflammation was measured by using the gingival index (GI) (12). This index is based on subjective but standardized criteria associated with clinical signs of color change, edema, and bleeding. The scale also consists of integers ranging from 0 to 3. PD and AL were chosen as standard measurements routinely utilized to quantitate the severity of periodontal disease (19). Both of these values were measured to the nearest whole millimeter with standardized and calibrated color-coded periodontal probes (University of Michigan "O" probe with the demarcations of Williams; Hu-Friedy Manufacturing Co., Chicago, Ill.) PD was recorded as the distance between the free gingival margin and apical extent of subgingival probe penetration. AL was recorded as the distance between the cemental-enamel junction and the apical extent of probe penetration.

All clinical measurements and indices were recorded at the mesio-buccal and mesio-lingual interproximal surfaces of the maxillary first molars by using the contact point with the adjacent tooth as a reference point for standardizing the measurement location.

Before initiation of this study, and at regular intervals throughout its duration, three examiners were trained in recording clinical data and the measurements they obtained were calibrated. All GI, PD, and AL values were recorded by two of the three investigators acting independently and blind to the observations of each other. Perfect interexaminer agreement between any two of the three clinical examiners averaged 74.4% for the GI. The average interexaminer agreement within +/− 1.0 mm was 98.6% for PD and 89.7% for AL. These findings were based on calibration testing involving 240 sites in 120 patients. Results of these calibration trials are comparable to those reported by others in similar calibration trials (5, 23). To increase the accuracy of clinical data, however, the GI was taken as the consensus value of the two independent examiners. If agreement occurred, that became the consensus value. If disagreement occurred, then the examiners rescored the site to obtain a mutually agreed-upon value. PD and AL values were averaged between the two examiners. All clinical values analyzed in this investigation consisted of the averages of values at the mesio-buccal and mesio-lingual surfaces of the respective teeth.

Bacterial cultivation and enumeration. Plaque samples were initially dispersed by repeated passage through a 25-gauge needle. A 50-μl portion of the sample was then removed from the anaerobic chamber and immediately counted with dark-field microscopy by a modified method of Listgarten to enumerate cocci, rods, curved rods, fusiforms, spirochetes, motile rods, and total bacterial forms (11). A minimum of one Y square of the Petroff-Hauser chamber area was counted, or additional whole Y squares, until a total of 100 bacterial forms per sample was reached. The portion of sample remaining was diluted with 2.0 ml of PRAS diluent and then sonified for 20 s (Kontes Cell Disruptor; Kontes, Vineland, N.J.). After sonication, the sample was serially diluted and then plated on various media by using a Spiral plater (Spiral Systems, Inc., Cincinnati, Ohio). Total cultivable anaerobic microbial flora and dark-pigmented *Bacteroides* spp. were enumerated on a supplemented blood agar media consisting of Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), 5% defibrinated sheep blood, 0.00005% menadione, and 0.0005% hemin (Sigma Chemical Co., St. Louis, Mo.), and incubated anaerobically at 37°C for 7 days. *Haemophilus* spp. were enumerated on chocolate agar modified from the work of Sims (20). This modified chocolate (MC) agar was formulated of boiled 9% Protease no. 3 agar (Difco Laboratories, Detroit, Mich.) and 2% Bacto-hemoglobin (Difco), autoclaved separately, mixed, and supplemented with 0.001% NAD, 0.001% hemin, 10 U of bacitracin per ml, and 0.0005% cloxacillin (Sigma). The MC agar was incubated in 10% CO_2 at 37°C for 3 days. *Streptococcus* spp. were enumerated on mitis-salivarius agar (Difco) supplemented with 0.1% potassium tellurite (Difco), incubated anaerobically at 37°C for 2 days, followed by 24 h at room temperature aerobically, and speciated as *Streptococcus sanguis*, *Streptococcus mutans*, and total streptococci by colonial morphology (1). Proportions of dark-pigmented *Bacteroides* spp., *Streptococcus* spp., and total *Haemophilus* spp. were based on the total counts of anaerobic cultivable flora enumerated from the supplemented blood agar. Proportions of total spirochetes were calculated based on the total direct microscopic count.

Identification of *Haemophilus* spp. After incubation of the MC agar, plates with the dilution that yielded between 10 and 40 discrete colonies were used so that every colony on the chosen plate was speciated. In 14 subjects, each colony from 14 supragingival and the corresponding subgingival plaques from a maxillary first molar was subcultured on MC agar and then speciated according to a scheme presented in Table 1. Cell morphology and gram reaction were determined from Gram-stained smears of 48-h cultures on MC agar. Determination of the haemin requirement (X factor) and NAD requirement (V factor) was done by a modified method of Sims (20) by streaking the isolates on plates of brain heart infusion agar (Difco) supplemented with either 0.001% haemin or 0.001% haemin and 0.001% NAD (Sigma). Production of ornithine decarboxylase was measured in decarboxylase basal broth (Difco) with 1.0% ornithine (Sigma) by the method of MacFaddin (13). The presence of the enzyme α-glucosidase was determined by the method of Laughon et al. (10). The presence of the enzyme β-galactosidase was measured by the procedure of MacFaddin (13). The ability to ferment a 1.0% solution of the carbohydrates glucose, sucrose, lactose, and xylose was tested in phenol

TABLE 1. Identification of dental plaque isolates from MC agar[a]

Organism[b]	NAD requirement[c]	Glucose	Lactose	Sucrose	Xylose	Ornithine decarboxylase	β-Galactosidase	α-Glucosidase
H. parainfluenzae	+	+	−	+	−	+/−[d]	d+	−
H. aphrophilus	−	+	+	+	−	−	+	d+
H. paraphrophilus	+	+	+	+	d−	−	+	d+
H. segnis	+	w	−	w	−	−	d	−
E. corrodens	−	−	−	−	−	+	−	−
A. actinomycetemcomitans	−	+	−	−	d−	−	−	−

[a] Symbols: +, 95 to 100% positive; d+, 75 to 95% positive; d, 25 to 75% positive; d−, 5 to 25% positive; −, 0 to 5% positive; w, weak reaction.
[b] Organisms identified as to species according to Kilian (7).
[c] Modified method of Sims (4).
[d] *H. parainfluenzae* biotypes I+ and II+, biotype III−, based on the results of Kilian and Schiott (9).

red broth (Difco). Incubation was for 7 days in 10% CO_2, the pH was measured, and a change of 0.5 U was recorded as positive. Proportions of each species per sample were based on the total number of colonies from a MC agar plate identified as being from the genus *Haemophilus*, *Actinobacillus*, or *Eikenella*.

RESULTS

Prevalence and proportions of *Haemophilus* spp. in dental plaque from 284 young adults. Haemophili were isolated from 527 of 535 supragingival sites and from 515 of 535 corresponding subgingival sites, resulting in prevalences of 98.5 and 96.2%, respectively. This genus appeared to be ubiquitous in both supragingival and subgingival plaque. Data were analyzed from 284 subjects or 1,070 supragingival and subgingival sites associated with 535 teeth. Thirty-three subjects had data for only one tooth (11.6%), and 251 subjects had data for both teeth (88.4%).

Proportional data for total *Haemophilus* spp., based on total anaerobic cultivable flora from supplemented blood agar, were computed for 284 subjects, sorted into decile ranks by listing all proportions in order from smallest to largest, and dividing the list or distribution for the 535 teeth (sites) into 10 approximately equal groups (Table 2). Fifty percent of the supragingival sites had 1.51% or fewer *Haemophilus* spp. Thirty percent had greater than 1.51% but less than 5.91% *Haemophilus* spp., and 20% had greater than 5.91% *Haemophilus* spp. The distribution of subgingival proportions for *Haemophilus* spp. was lower. Fifty percent of the subgingival sites had 0.33% or less, 70% had 1.36% or less, and 20% had greater than 1.36% but less than 5.47%. Only 10% exhibited 5.47% or more. The median proportion of *Haemophilus* spp. was 1.51% supragingivally and 0.33% subgingivally. In addition, supragingival plaque had higher proportions of *Haemophilus* spp. than subgingival plaque from the same tooth in 74.6% of the cases (data not shown), with the converse being true in the remaining 25.4%.

Localization of *Haemophilus* species compared with other species at the same sites. Each of the sites from which plaque was collected presented a unique mixture of genuses and species of microorganisms. The relationship of proportions of one genus to those of another at the same supragingival or subgingival sites was examined. Scatter diagrams were drawn by plotting proportions of total *Haemophilus* spp. for each site examined from each maxillary first molar on the y axis and proportions of streptococci, dark-pigmented *Bacteroides* spp., or spirochetes on the x axis (Fig. 1). The proportions were separated into those from supragingival and subgingival plaque samples. Approximately half of the data points were randomly deleted from the diagrams to clarify the illustrations.

The bivariate distributions of proportions of haemophili with proportions of streptococci in supragingival and subgingival sites are illustrated in Fig. 1A and B, respectively. These organisms are ubiquitous, and they inhabit the same sites. Both organisms tend to occur in lower proportions in subgingival rather than in supragingival sites. In either case, however, the streptococci were usually in higher proportions than the haemophili. All levels of haemophili were associated with all levels of streptococci.

The bivariate distributions of proportions of hemophili with dark-pigmented *Bacteroides* spp. clearly differed from those with streptococci (Fig. 1C and D). Much of the data associated with dark-pigmented *Bacteroides* spp. was clustered in the lower-left-hand corner of the diagram (<8% *Bacteroides* spp. and <4% haemophili). Many data points appeared along axes associated with low proportions of the respective organisms. Little data appeared in the body of the diagram. High proportions of haemophili were virtually never associated with high proportions of *Bacteroides* spp., and, conversely, high proportions of *Bacteroides* spp. were virtually never associated with high proportions of haemophili. This indicated that these bacterial groups did not tend to occupy the same sites. In addition, haemophili generally were recovered more frequently and in higher proportions than *Bacteroides* spp. in supragingival plaques, and *Bacteroides* spp. were recovered in higher proportions in subgingival plaques.

The bivariate distributions of haemophili with spirochetes were very similar to those observed with dark-pigmented

TABLE 2. Decile ranks of total *Haemophilus* spp. proportions[a] in supragingival and subgingival plaque from 535 maxillary first molars from 284 young adults

Decile rank[b]	Supragingival (%)	Subgingival (%)
1st	0–0.062	0–0.0092
2nd	0.063–0.215	0.0093–0.038
3rd	0.216–0.420	0.039–0.089
4th	0.421–0.770	0.090–0.172
5th	0.771–1.51	0.173–0.330
6th	1.52–2.27	0.331–0.730
7th	2.28–3.35	0.731–1.36
8th	3.36–5.91	1.37–2.53
9th	5.92–12.0	2.54–5.46
10th	12.1–100.0	5.47–100.00

[a] Total *Haemophilus* spp. proportions were based on total anaerobic cultivable flora from supplemented blood agar (×100).
[b] Plaque samples were divided into decile ranks by listing the total *Haemophilus* spp. proportions from the smallest to the largest value for supragingival and subgingival samples and dividing the lists into 10 approximately equal groups.

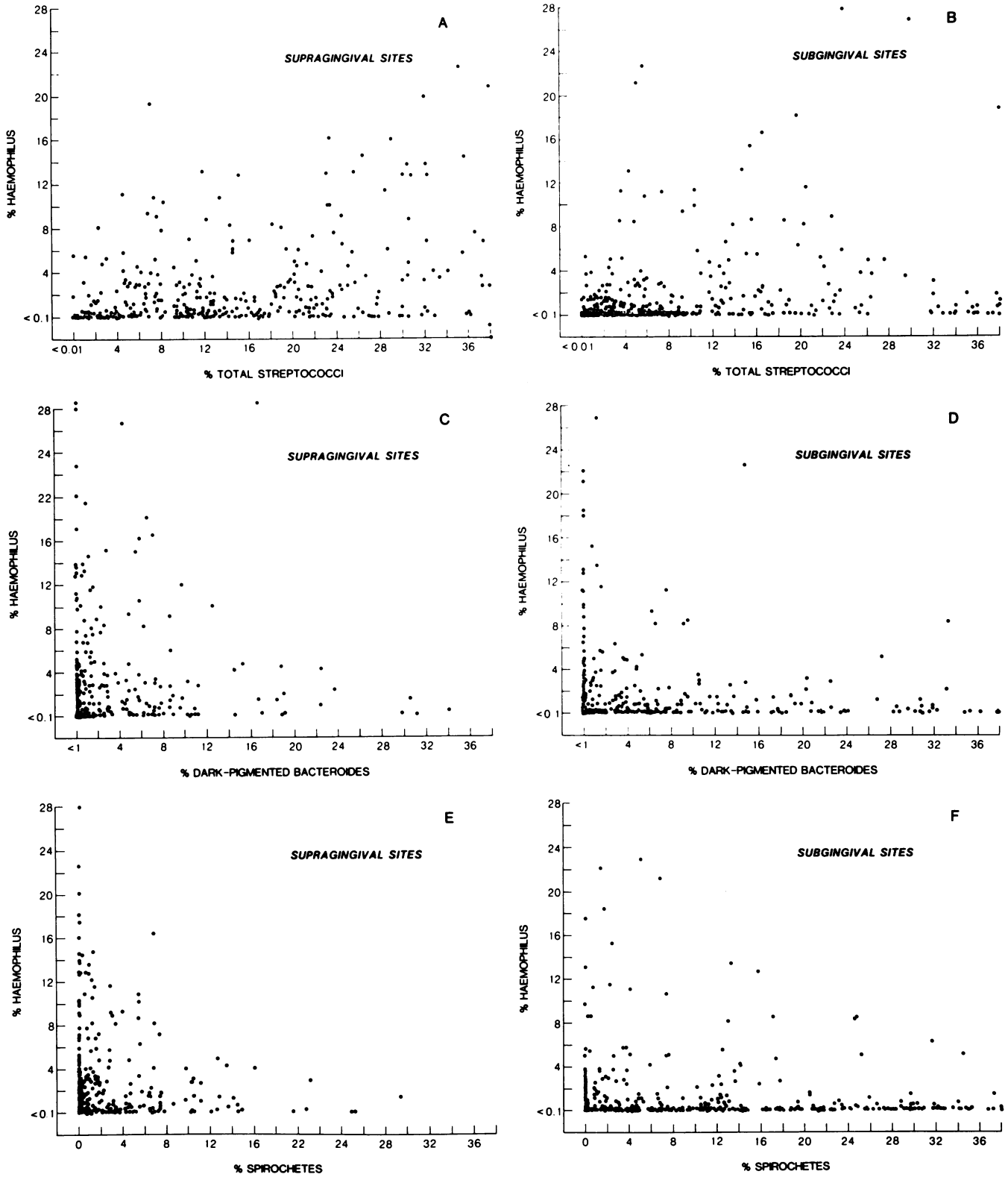

FIG. 1. Bivariate distributions of proportions of total *Haemophilus* spp. with proportions of streptococci, dark-pigmented *Bacteroides* spp., or spirochetes for each supragingival or subgingival site examined.

Bacteroides spp. (Fig. 1E and F). Haemophili were in higher proportions than spirochetes in supragingival plaques, and spirochetes were in higher proportions than haemophili in subgingival plaques. Here again, high proportions of haemophili were virtually never associated with high proportions of spirochetes, and conversely, high proportions of spirochetes were virtually never associated with high proportions of haemophili.

Distribution of *Haemophilus* species identified in samples from 14 subjects. A total of 377 isolates from MC agar from the upper maxillary molars of 14 subjects were further examined by the criteria given in Table 1. A total of 408 colonies were isolated. The 377 colonies surviving subculture (92.4%) were conclusively speciated. Seventy-two percent (270) of the surviving 377 isolates required NAD, with 107 (28%) of the isolates independent of NAD and identified as *Haemophilus aphrophilus*, *Eikenella corrodens*, or *Actinobacillus actinomycetemcomitans*. All other isolates were assigned as *Haemophilus parainfluenzae*, *Haemophilus segnis*, and *Haemophilus paraphrophilus*.

The prevalence of these species in subjects is given in Table 3. *H. parainfluenzae* was the most prevalent, found in 12 (86%) and 10 (71%) of the 14 supragingival and subgingival sites, respectively. *H. aphrophilus* was found in seven (50%) of the subgingival sites versus four (29%) of the supragingival sites. *H. aphrophilus* was never found in the supragingival plaque of a tooth unless it was also found subgingivally (data not shown). A similar number of subjects were found to have *H. paraphrophilus* and *H. segnis* in the supragingival and subgingival sites. Both *A. actinomycetemcomitans* and *E. corrodens* were found on the MC agar from 2 of the 14 subgingival plaque samples.

Description of the periodontal status of 535 teeth from 284 young adults. The status of the supporting tissues surrounding the right and left maxillary first molars was determined. Clinical measurements for PD, AL, PI, and GI were sorted into decile ranks by listing all measurements in order from smallest to largest and dividing the list or distribution for the 535 teeth (sites) into 10 approximately equal groups (Table 4). Approximately 10% of the teeth had a PD of ≤2.25 mm. PDs were ≤3.0 mm for ca. 50% of the sites. The next ca. 30% of the PDs ranged in value from 3.25 to 3.75 mm. Twenty percent of the teeth had a PD of ≥4.00 mm, whereas 10% had a PD of ≥5.0 mm. Only 15 teeth had a PD of ≥7.00 mm. ALs were 0 mm for ca. 20% of the teeth. ALs ranged from 0.25 to 1.00 mm for 50% of the teeth, 1.25 to 1.50 mm for 10%, 1.75 to 2.50 mm for 10%, and 2.75 to 9.50 mm for 10%. Approximately 10% of the PI measurements were ≤0.5, whereas ca. 50% were ≤1.0. Approximately 30% of the PI values were ≥2.0. The GI was ≤1.5 for ca. 20% of the teeth, 1.75 for ca. 20%, and ≥2.0 for the remaining 60%.

Relationships between clinical parameters and proportions of total *Haemophilus* spp. found in supragingival and subgingival plaques. Upon inspection of the data from 535 individual maxillary upper first molars, it appeared that an inverse relationship existed between PD or AL and the proportions of total *Haemophilus* spp. That is, when proportions of *Haemophilus* spp. were high in either supragingival or subgingival plaque, the PD or AL was low (data not shown). Also, in sites where PD or AL was high, the proportions of total *Haemophilus* spp. were low. The Tau correlation coefficients of Kendall were computed for the relationships between the proportions of total *Haemophilus* spp. and the PI, GI, PD, and AL (Table 5). Statistically significant correlations occurred between proportions of total haemophili and the GI, PD, and AL with both supragingival and subgingival plaque samples. Although the inverse relationship that was apparent by inspection turned out to be a real one, the correlation coefficients were very small.

Relationships between PDs and proportions of specific *Haemophilus* species from the plaques from 14 subjects. The relationship between haemophili and the status of the periodontium was also studied by analyzing data associated with the distribution of *Haemophilus* spp. identified from MC agar in the samples from 14 subjects. The overall proportions of each *Haemophilus* spp., as well as of *E. corrodens* and *A. actinomycetemcomitans*, from the 377 isolates speciated are shown in Table 6. Sites were divided on the basis of supragingival and subgingival samples and further separated by PD. Of the 14 maxillary first molars, 7 had a PD of ≤3.0 mm (mean PD = 2.9 mm), and the remaining 7 sites had a PD of >3.0 mm (mean PD = 5.0 mm). The overall proportions of the respective species were based on the sum of all colonies identified in the respective group of plaque samples, i.e.,

TABLE 4. Decile ranks of clinical measurements[a] from 535 maxillary first molars from 284 young adults

Decile rank[b]	PD (mm)	AL (mm)	PI	GI
1st	0–2.25	0	0–0.5	0–1.25
2nd	2.25–2.50	0	0.5–1.0	1.25–1.50
3rd	2.50–2.75	0–0.25	1.0	1.50–1.75
4th	2.75–3.00	0.25	1.0	1.75
5th	3.00	0.25–0.50	1.0	1.75–2.00
6th	3.00–3.25	0.50–0.75	1.0–1.5	2.00
7th	3.25–3.50	0.75–1.00	1.5	2.00
8th	3.50–3.75	1.25–1.50	1.5–2.0	2.00
9th	4.00–4.75	1.75–2.50	2.0	2.00
10th	5.00–9.25	2.75–9.50	2.0–3.0	2.00–3.00

[a] Measurements were taken by two examiners at the mesial-buccal and mesial-lingual surfaces of maxillary first molars and averaged for each site.
[b] Maxillary first molar sites were divided into decile ranks by listing the respective clinical values from smallest to largest and dividing the list into 10 groups of approximately equal size.

TABLE 3. Prevalence of *Haemophilus*, *Actinobacillus*, and *Eikenella* species isolated from supragingival and the corresponding subgingival plaque samples of maxillary first molars from 14 subjects

Organism	Prevalence (no. of samples)	
	Supragingival	Subgingival
H. parainfluenzae	12	10
H. aphrophilus	4	7
H. paraphrophilus	4	4
H. segnis	6	5
E. corrodens	3	2
A. actinomycetemcomitans	1	2

TABLE 5. Correlations[a] between proportions of total *Haemophilus* species and clinical measurements

Clinical measurement	Correlation coefficients	
	Subgingival	Supragingival
PI	−0.0452 (NS)[b]	−0.0097 (NS)
GI	−0.0845 ($P \leq 0.014$)[c]	−0.0746 ($P \leq 0.032$)[c]
PD	−0.1311 ($P \leq 0.002$)[c]	−0.0712 ($P \leq 0.028$)[c]
AL	−0.1463 ($P \leq 0.002$)[c]	−0.1063 ($P \leq 0.004$)[c]

[a] The Tau correlation coefficients of Kendall.
[b] NS, Not statistically significant.
[c] Statistically significant correlations with associated probability values.

TABLE 6. Overall proportions of *Haemophilus*, *Eikenella*, and *Actinobacillus* species in 377 isolates speciated from supragingival and corresponding subgingival plaque samples of maxillary first molars from each of 14 subjects, and the proportions with the same samples separated by PDs[a]

Organism[b]	Supragingival (%)[c]			Subgingival (%)[c]		
	All teeth	≤3.0 mm	>3.0 mm	All teeth	≤3.0 mm	>3.0 mm
H. parainfluenzae	54	62	44[d]	27	46	8
H. aphrophilus	11	12	10	35	16	55
H. paraphrophilus	17	20	12	18	28	8
H. segnis	14	6	27	10	10	10
E. corrodens	2	0	4	3	0	6
A. actinomycetem-comitans	2	0	4	7	0	14
Total no. of colonies	200	118	82	177	89	88

[a] Fourteen maxillary molars sampled were divided into two groups of seven teeth each based on probing depth of ≤3.0 mm and >3.0 mm.
[b] Colonies were speciated according to Kilian (7).
[c] The proportions are based on the total number of colonies speciated from the plaque samples, either supragingival or subgingival, and according to the respective PDs.
[d] Species paired in boxes show a statistically significant difference in the proportion of a species between shallow (≤3.0 mm) versus deeper (>3.0 mm) sites, by the Chi-square test ($P < 0.0005$).

supragingival sites from teeth with PDs of ≤3.0 mm had an overall proportion of 62% *H. parainfluenzae* (73 of the 118 colonies isolated from the seven supragingival sites associated with teeth with PDs of ≤3.0 mm were *H. parainfluenzae*).

The proportion of *H. parainfluenzae* was significantly higher in supragingival and subgingival sites associated with teeth with shallower PDs than in those associated with teeth with deeper PDs (Table 6, Chi-square test). Similarly, *H. paraphrophilus* was found in higher proportions in subgingival plaque from shallower sites (28% versus 8%). Alternatively, the proportion of *H. segnis* was significantly higher in supragingival sites with deeper PDs, and *H. aphrophilus* was significantly higher in subgingival sites with deeper PDs. *E. corrodens* and *A. actinomycetemcomitans* were only found at sites with deeper PDs in both supragingival and subgingival plaque.

Multiple relationships between proportions of total haemophili, proportions of specific *Haemophilus* species, and PDs. The finding of statistically significant correlations between proportions of total *Haemophilus* spp. and PDs (Table 5) and statistically significant relationships between proportions of specific *Haemophilus* spp. and PDs (Table 6) suggested these proportional microbial measures might be related to each other, as well as to the peridontal status of the sampling sites. This possibility was examined with data from the 14 sites conclusively speciated (Table 7). The 14 sites are listed in descending order based on the decile ranks of the proportions of total haemophili. There was considerable variation in the combinations of *Haemophilus* spp. in supragingival and subgingival plaques associated with the respective sites; however, higher proportions of *H. parainfluenzae* or *H. paraphrophilus*, or both, were often associated with higher decile ranks of proportions of total haemophili, and higher proportions of *H. aphrophilus* or *H. segnis*, or both, were often associated with lower decile ranks of proportions of total *Haemophilus* spp.

Predictions of relative PDs based on the decile ranks of proportions of total haemophili and on the proportions of specific *Haemophilus* spp. were used to test the statistical significance of the relationships between the total haemophili and the specific *Haemophilus* spp. proportions identified above (Table 7). Proportions of total haemophili were hypothesized to predict sites with the least PD when the decile rank of proportions of total haemophili was high in supragingival and subgingival plaque samples from the same site. The prediction of moderate PDs was based on those transitional plaques with low decile ranks of total haemophili proportions in supragingival samples and high decile ranks in

TABLE 7. Multiple relationships among proportions of total haemophili, proportions of specific *Haemophilus* species, and predicted PD categories[a]

Subject no.[b]	Predicted PD category		Decile rank of total *Haemophilus* spp.[c]	Proportion (%) of *Haemophilus* colonies by species[g]			
	Total[c]	Species[d]		*H. parain-fluenzae*	*H. paraphro-philus*	*H. aphro-philus*	*H. segnis*
320	Least	Least[f]	90 (90)	100 (100)	0 (0)	0 (0)	0 (0)
312	Least	Least[f]	80 (90)	85.7 (50.0)	4.8 (50.0)	0 (0)	9.5 (0)
311	Least	Least[f]	70 (90)	41.7 (40.0)	58.3 (60.0)	0 (0)	0 (0)
313	Least	Least[f]	70 (90)	0 (0)	100 (100)	0 (0)	0 (0)
323	Least	Least[f]	80 (80)	70.0 (11.1)	0 (0)	0 (0)	0 (0)
319	Moderate	Greatest	40 (80)	40.0 (0)	0 (0)	9 (93.6)	60.0 (6.3)
330	Moderate	Moderate[f]	30 (80)	83.3 (35.7)	0 (0)	16.7 (64.3)	0 (0)
326	Moderate	Greatest	20 (80)	12.5 (0)	0 (0)	87.5 (100)	0 (0)
310	Moderate	Moderate[f]	30 (70)	62.2 (62.5)	0 (0)	35.1 (31.3)	2.7 (6.3)
329	Moderate	Moderate[f]	30 (60)	63.1 (57.1)	0 (0)	0 (0)	36.8 (28.6)
315	Greatest	Greatest[f]	60 (30)	55.6 (14.3)	0 (0)	0 (85.7)	0 (0)
316	Greatest	Moderate	50 (30)	17.7 (0)	58.8 (46.7)	0 (13.3)	17.7 (40.0)
331	Greatest	Greatest[f]	40 (10)	66.7 (42.9)	0 (0)	0 (0)	33.3 (57.1)
324	Greatest	Greatest[f]	0 (30)	0 (8.3)	0 (0)	25.0 (25.0)	0 (0)

[a] Data associated with supragingival and subgingival plaque are shown. Data for subgingival plaque are in parentheses.
[b] Subjects were ordered on the basis of the sums of decile ranks of total *Haemophilus* spp. in supragingival and subgingival plaque samples. In cases of equal sums, the subject with the highest subgingival rank is listed first.
[c] Relative PDs are predicted to increase as decile ranks of proportions of total *Haemophilus* spp. decrease: Least depth equals high *Haemophilus* ranks in both supragingival and subgingival plaques, moderate depth equals low *Haemophilus* ranks in supragingival plaques and high ranks in subgingival plaques, and greatest depth equals low *Haemophilus* ranks in subgingival or in supragingival and subgingival plaques.
[d] High proportions of *H. parainfluenzae* or *H. paraphrophilus*, or both, predict the least PD, and low proportions predict the greatest PD. Moderate to high proportions of *H. aphrophilus* or *H. segnis*, or both, also predict the greatest PD. Moderate PDs are predicted when the preceeding conditions do not reconcile.
[e] Limits for decile ranks are tabulated in Table 2.
[f] Exact agreement between the respective hypothesized microbial systems for assessing disease risk 11 of 14 times (78.6%) is statistically significant ($P \leq 0.001$).
[g] The proportions of each *Haemophilus* spp. based on the number of colonies identified as to species from each supragingival or subgingival sample of a subject.

subgingival samples, and the prediction of the greatest PDs occurred when the decile ranks were low in subgingival or in subgingival and supragingival samples from the same sites. In a similar manner, high proportions of *H. parainfluenzae* or *H. paraphrophilus*, or both, were hypothesized to predict the least PDs, and low proportions were hypothesized to predict the greatest PDs. Moderate to high proportions of *H. aphrophilus* or *H. segnis*, or both, also predicted the greatest PDs. Moderate PDs were predicted when the preceding conditions did not reconcile.

The relationship between predicted PD categories based on decile ranks of proportions of total haemophili and proportions of specific *Haemophilus* spp., respectively, was examined. Exact agreement occurred between the respective techniques 11 of 14 times (78.6%). This agreement was statistically significant ($P \leq 0.001$) based on Fischer's exact test and on an exact test of significance of a binomial proportion when chance agreement would be expected only one of three times (33.3%).

The validity of these microbial predictors of PD categories was examined by comparing the mean PD associated with sites in the respective prediction categories (Table 8). In all cases the mean PD increased as the respective PD categories increased. The F statistic testing for a difference in PDs between prediction categories was statistically significant ($P \leq 0.01$) for the decile ranks of the total haemophili technique. The F statistic for the specific *Haemophilus* spp. technique was not statistically significant. Because of the relatively small sample sizes in each of the risk categories, however, statistical significance would have also been obtained with the proportion of *Haemophilus* spp. technique if a single site (subject 316, Table 7) had been classified as greatest instead of moderate.

TABLE 8. Mean PDs associated with PD predictions based on decile ranks of proportions of total haemophili and specific *Haemophilus* species

PD prediction category	Technique			
	Decile rank of total *Haemophilus* spp.		Specific *Haemophilus* spp.	
	Mean PD (mm)	No. of samples	Mean PD (mm)	No. of samples
Least	3.05	5	3.05	5
Moderate	3.35	5	4.31	4
Greatest	5.88	4	4.60	5
F statistic[a]	8.157 ($P \leq 0.01$)[b]		1.317 (NS)[c]	

[a] This is the F statistic with 2 and 11 degrees of freedom, testing for a difference in PDs among risk categories (separate one-way analysis of variance for each hypothesized technique.)

[b] Probability value for a statistically significant difference in PDs among prediction categories.

[c] There was no statistically significant difference in PDs among prediction categories.

DISCUSSION

This study represents an overview of the microecology of the genus *Haemophilus* in supragingival and subgingival plaque samples from a large number of peridontally near-healthy maxillary first molars in young adults. Young adults were selected to increase the likelihood of observing a range of periodontal conditions, including the transition between health and early periodontitis. Focusing attention at this point in the pathogenic continuum provides potential opportunity for impacting upon methods for preventing disease or making early diagnosis or assessments of disease risk, or both. Maxillary first molars were selected for sampling sites because there is evidence that they are among the first teeth to present with disease (4, 14, 15).

Data summarizing the clinical status of subjects in this study was presented in Table 4. The term near-healthy is an appropriate description of the sample sites, since relatively few sites presented with indications that significant breakdown of periodontal support had taken place. Only 30% of the sites had a >1.0-mm AL. Only 11 sites (4%) had notable signs of disease, with a GI of >2.0, a PD of >6.0 mm, and an AL of >4.0 mm (data not shown). Alternatively, only 15 sites (5%) had signs of health, with a GI of <1.0, a PD of <3.0 mm, and an AL of <1.5 mm. Although no attempt was made to document active disease, it is reasonable to expect that active disease was present among some of the 284 subjects.

The genus *Haemophilus* was recovered from nearly every supragingival and subgingival site examined. This finding was similar to the earlier studies by Sims (21), who examined samples of saliva and gingival fluid, and by Kilian (7), who examined numerous samples of human saliva. Both investigators found the oral *Haemophilus* species in nearly every sample examined. In a further study, Kilian and Schiott also cultivated, enumerated, and speciated *Haemophilus* spp. from 15 plaque samples (9). No description of the periodontal status of the subjects or the sampling technique used to obtain plaque samples was provided. Their samples were probably supragingival or pooled supragingival and subgingival plaques, or both. Although the bacteria were not cultivated under identical conditions, Kilian (7) found a mean proportion of *Haemophilus* spp. of 2%, which is similar to the median proportions found in this study (Table 2). Although oral haemophilii are ubiquitous, they often occur in small proportions. It is essential, therefore, that sampling and culturing techniques capable of identifying microorganisms occurring in small proportions be used when cutivating the oral haemophili.

Numerous other studies reported on the cultivable microflora of plaque in adult and juvenile periodontitis (3, 16, 17, 22, 24). Few of these studies included the genus *Haemophilus*. This could be because most of the studies employed a supplemented blood agar media that would not support, or would only poorly support, the growth of most *Haemophilus* species.

The use of MC agar as a selective medium to enumerate the genus *Haemophilus* as a whole was found to be useful, but somewhat limited by the different species that grew on it. Recent studies indicated that *H. aphrophilus* and *H. paraphrophilus* are closely related genetically, but the differing NAD requirement has kept their appearance as a single species uncertain (2, 18, 25). Similarly, these same studies suggested that *H. aphrophilus* and *A. actinomycetemcomitans* are separate species that belong in the same genus due to their genetic relatedness, as well as their lack of a requirement for NAD. Only one study included information concerning *H. parainfluenzae* as related to the other oral haemophili, and it suggested that this species is genetically less related than the other oral *Haemophilus* species (18). Tables 3 and 6 indicate that all of these species, probably representing two related but separate genuses, will grow on MC agar. The PDs from which plaque samples were selected, therefore, appear to have some bearing upon the specific *Haemophilus* species accounting for the total haemophili recovered on MC agar (Table 6).

Recently two laboratories reported on the relationship between the genus *Haemophilus* and the status of the periodontium. Moore et al. (16) reported that at sites with severe periodontitis *H. segnis* and *H. paraphrophilus* were found only in the residual supragingival flora and not in the subgingival flora. Alternately, Slots and co-workers found *H. segnis* in subgingival plaque from pockets of ≥6.0 mm in subjects with adult periodontitis (J. Slots, personal communication). Our subjects probably presented with less severe disease than those studied by Moore et al. and Slots and coworkers. However, there appear to be similarities between our respective data for *H. segnis*. In our laboratory *H. segnis* was found in subgingival plaques. In supragingival plaques, however, it was found in significantly greater proportions in those sites associated with PDs of >3.0 mm. These different but related findings may be associated with changes in the microbial composition of plaque which start in supragingival plaque and progress into subgingival plaque as the status of the periodontium changes from health to early disease to advanced disease. The relationship that we found between the decile ranks of proportions of total *Haemophilus* spp. in supragingival and subgingival plaques and the proportions of species isolated on MC agar is consistent with this concept of sequential change. The finding of somewhat different microhabitats of the *Haemophilus* spp. between laboratories requires further reconciliation, however, and suggests the need for additional study of the relationships of separate species to periodontal health and disease.

Scatter diagrams have been very useful in clarifying the complex ecologic patterns among microorganisms in their microhabitats around the teeth. The bivariate distribution of total *Haemophilus* spp. with streptococci illustrated that all levels of total *Haemophilus* spp. occurred with all levels of streptococci. Similar to the work of Kilian et al. (8) however, the proportions of *Haemophilus* spp. tended to be lower than the proportions of streptococci at the same sites. The bivariate distributions of haemophili with dark-pigmented *Bacteroides* spp. and with spirochetes clearly presented a different picture.

Dark-pigmented *Bacteroides* spp. and spirochetes have been associated with periodontal disease (11, 22). We reported a negative correlation between proportions of total *Haemophilus* spp. and PD (Table 5) and proposed that high proportions of total *Haemophilus* spp. are associated with minimal PDs (Table 7). We might in turn infer that total *Haemophilus* spp. are associated with health. Scatter diagrams have shown that high proportions of haemophili were virtually never recovered from the same sites as high proportions of *Bacteroides* spp. or spirochetes. Conversely, high proportions of *Bacteroides* spp. or spirochetes were virtually never recovered from the same sites as high proportions of haemophili. The finding of similar bivariate distributions of haemophili with *Bacteroides* spp. and with spirochetes was not a surprise. *Bacteroides* spp. and spirochetes are anaerobes. They may be expected, therefore, to occupy different sites than the facultatively anaerobic *Haemophilus* species. This does not imply, however, that dark-pigmented *Bacteroides* spp. and spirochetes necessarily occupied the same sites. Although this was true in some cases, it was also true, as in the case of haemophili and streptococci, that all levels of *Bacteroides* spp. were recovered from sites with all levels of spirochetes (data not shown).

Data obtained in this investigation demonstrated that relationships exist between oral haemophili and the status of the periodontium. Two potential systems for interpreting haemophili data were hypothesized for predicting probing depths. It is possible that these systems may in turn be used in future investigations for assessing the risk of periodontal disease. Although these systems may have practical implications, they were proposed primarily for purposes of interpreting data in this study. Specifically, they demonstrated that there was a relationship between the proportions of total *Haemophilus* spp. in supragingival and subgingival plaque and the proportions of specific *Haemophilus* spp. in the same samples (Table 7). High proportions of *H. parainfluenzae* and *H. paraphrophilus* were associated with high proportions of total *Haemophilus* spp., and high proportions of *H. segnis* and *H. aphrophilus* were associated with low proportions of total *Haemophilus* spp. This finding was confirmed by the highly significant agreement between hypothesized prediction of relative PD categories based on proportions of total *Haemophilus* spp. and prediction of relative PD categories based on the set of proportions of specific *Haemophilus* species.

The hypothesized system based on proportions of specific *Haemophilus* spp. also demonstrated the importance of interpreting plaque in terms of multiple species. Plaque samples from subjects 320, 312, 311, and 313 in Table 7, for example, all had similarly high proportions of total *Haemophilus* spp. and were consequently classified in the least PD prediction category. *H. parainfluenzae*, however, accounted for 100% of the total *Haemophilus* spp. in supragingival and subgingival plaque samples from subject 320, and *H. paraphrophilus* accounted for 100% of the total *Haemophilus* spp. from subject 313. Combinations of *H. parainfluenzae* and *H. paraphrophilus* accounted for essentially all of the total *Haemophilus* spp. in subjects 312 and 311. Even though there was no apparent relationship between *H. parainfluenzae* or *H. paraphrophilus* individually and the proportions of total *Haemophilus* spp., when the entire set of proportions of specific *Haemophilus* spp. was examined, virtually all of the *Haemophilus* spp. collected from sites predicted to have the least PD consisted of *H. parainfluenzae* or *H. paraphrophilus*, or both.

With proportions of some *Haemophilus* spp. increasing while others decrease as the proportions of total *Haemophilus* spp. to total anaerobic cultivable flora change, it was not surprising to find low correlation coefficients for relationships between proportions of total *Haemophilus* spp. and clinical signs of disease. Those correlations were probably also low because they were based on cross-sectional (static) data associated with dynamic relationships. On the other hand, consistency between the two hypothesized systems for predicting PDs based upon oral haemophili (Table 7), as well as the increasing mean PDs associated with the respective PD prediction categories (Table 8), were encouraging findings with respect to the role that haemophili might eventually play in the microbial interpretations of the status of the periodontium or the risk of periodontal disease.

Developing a microbial system for interpreting periodontal status will ultimately require a longitudinal study examining the dynamic relationships between a set of oral microorganisms and between those organisms and the periodontium. If emphasis is placed on changes associated with the transition from health to early disease, then the genus *Haemophilus* should be among the microorganisms studied, and both supragingival and subgingival plaque should be analyzed. Sufficient subjects and sites must be included to assure good estimates of the distributions of multiple relationships among microorganisms and between microorganisms and periodontal health or disease.

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